The caspase proteolytic system in callipyge and normal lambs in longissimus, semimembranosus, and infraspinatus muscles during postmortem storage

C. M. Kemp, D. A. King, S. D. Shackelford, T. L. Wheeler and M. Koohmaraie

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The caspase proteolytic system in callipyge and normal lambs in longissimus, semimembranosus, and infraspinatus muscles during postmortem storage[1]

C. M. Kemp, D. A. King, S. D. Shackelford, T. L. Wheeler,[2] and M. Koohmaraie[3]

Roman L. Hruska US Meat Animal Research Center, USDA, ARS, Clay Center, NE 68933-0166

ABSTRACT: The objective of this experiment was to determine whether the caspase proteolytic system has a role in postmortem tenderization. Six ewes and 6 wethers that were noncarriers and 6 ewes and 6 wethers that were expressing the callipyge gene were used for this study. Caspase activities were determined in LM at 7 different time points during the postmortem storage period: 0 h, 4 h, 8 h, 24 h, 2 d, 7 d, and 21 d and in semimembranosus (SM) and infraspinatus (IS) muscles at 0 h, 8 h, 24 h, and 7 d from callipyge and noncallipyge (normal) lambs. Calpastatin activity was determined at 0 h, 2 d, 7 d, and 21 d and slice shear force measured at 2, 7, and 21 d in the LM. Calpastatin activity and slice shear force were greater in LM from callipyge lambs than normal lambs at each time point ($P < 0.001$ and $P < 0.0001$, respectively). Caspases 3 and 7 are executioner caspases, and their combined activity was found to decrease during the postmortem storage period in LM, SM, and IS muscles from callipyge and normal lambs. Similarly, activity of the initiator caspase (caspase 9) decreased ($P < 0.05$) in all 3 muscles across the postmortem storage period in callipyge and normal lambs, and its decrease in activity preceded that of the executioner caspases 3/7. A positive relationship also was detected between caspase 9 and caspase 3/7 in LM, SM, and IS muscles ($P < 0.0001$, r = 0.85, r = 0.86, r = 0.84, respectively), which is consistent with caspase 9 being responsible for the cleavage and activation of the executioner caspases (caspase 3/7) downstream. Caspase 3/7 and caspase 9 activities at 8 h in SM were greater in normal lamb than callipyge lamb ($P < 0.05$), with a trend for caspase 3/7 activity to be greater at 24 h postmortem ($P = 0.0841$). There also was a trend for caspase 3/7 activity to be greater in LM at 21 d in normal lamb than in callipyge lamb ($P = 0.053$), although there were no differences detected in caspase activities between genotypes in the IS muscle, which is not affected by the callipyge gene. A negative relationship also was detected between peak caspase 3/7 activity at 8 h in LM from normal lambs and calpastatin activity at 0 and 2 d (r = −0.65, r = −0.68, respectively, $P < 0.05$). This relationship was not observed in LM from callipyge lambs, suggesting that caspase 3/7 may be cleaving calpastatin in normal lambs but the level of calpastatin in callipyge lambs is such that caspase 3/7 cannot degrade it sufficiently to overcome the increased content of calpastatin, and thus, calpastatin activity is the overriding factor in postmortem proteolysis in these animals. There was no direct evidence from this study that caspases have a significant role in postmortem tenderization, but they may have some role through calpastatin degradation.

Key words: callipyge, calpastatin, caspase, proteolysis, tenderness

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INTRODUCTION

Caspase research has predominantly focused on their involvement in programmed cell death, functioning as vital executioners of apoptosis. However, caspases have been identified as a potential contributor to postmortem tenderization, cleaving several myofibrillar proteins that also are cleaved by calpains such as desmin, troponin T, troponin I, and spectrin (Kemp and Parr, 2008). In addition to the substrates that caspases cleave, there is growing evidence of interactions between the calpain and the caspase systems. Calpains have been shown to cleave caspases in both a pro- and anti-apoptotic

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[2]Corresponding author: Tommy.Wheeler@ars.usda.gov

[3]Present address: IEH Laboratories and Consulting Group, Lake Forest Park, WA 98155.

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manner (Chua et al., 2000; Nakagawa and Yuan, 2000). Calpastatin is a known caspase substrate, with cleavage by caspases 3 and 7 producing distinct fragments (Wang et al., 1998). Neumar et al. (2003) showed that human neuroblastoma cells overexpressing calpastatin not only inhibited calpain activity but also upregulated caspase activity and accelerated apoptosis.

Sheep carrying the callipyge gene are characterized by heavy muscling and decreased meat tenderness (Koohmaraie et al., 1995; Freking et al., 1999). Muscle enlargement in callipyge animals is primarily due to myofiber hypertrophy, which develops after about 3 wk of age (Jackson et al., 1997a). Callipyge lambs exhibit several desirable production characteristics including larger LM areas and superior lean composition (Jackson et al., 1997a,b). However, callipyge lambs have decreased meat tenderness, which is associated with greater calpastatin content compared with normal lambs (Koohmaraie et al., 1995, Delgado et al., 2001), resulting in unacceptable consumer tenderness ratings (Kerth et al., 2003). The effect of the callipyge phenotype on calpastatin activity and muscle hypertrophy varies among muscles with some muscles (e.g., infraspinatus, **IS**) not hypertrophied and others hypertrophied to a great extent (e.g., semimembranosus, **SM**). Callipyge sheep provide a useful model for studying a protease system in postmortem proteolysis because there are large differences in tenderness between muscles from callipyge and normal lambs. The objective of this experiment was to determine whether the caspase proteolytic system has a role in meat tenderization by determining caspase activity during postmortem storage of callipyge and normal lamb LM, IS, and SM.

MATERIALS AND METHODS

All animal procedures were reviewed and approved by the US Meat Animal Research Center Animal Care and Use Committee, and procedures for handling sheep complied with those specified in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1999).

Animals and Muscle Sampling

Twenty-four lambs (12 ewes and 12 wethers) of approximately 14 mo of age were used for this experiment. Six ewes and 6 wethers were expressing the callipyge phenotype, and 6 ewes and 6 wethers were noncallipyge. Animals were determined to be expressing the callipyge phenotype or not by visual assessment of muscle hypertrophy in the hind limbs. Within 30 min postexsanguination, approximately 2-g samples of LM (5th lumbar vertebra region), IS, and SM muscle were taken from the left side of the carcass (located such that a second sample would leave 2 equally spaced sample locations across the longest dimension of the muscles) and snap frozen in liquid nitrogen for subsequent caspase activity assays. In addition, approximately 10 g of LM was collected for calpastatin activity from the same location as the caspase sample. Carcasses were then hung in the chiller at 4°C, and 2-g samples of LM were taken at 4, 8, and 24 h postmortem from the left side of the carcass at locations 5 cm anterior to the most recent sample. Samples of IS and SM were taken at 8 h from the left side of the carcass and at 24 h from the right side and snap frozen in liquid nitrogen. At 48 h postmortem, the entire LM, IS, and SM were removed from the right side of the carcass. A 23-cm-long section of LM was cut from the center at the largest portion of the muscle (approximately 9th rib to 4th lumbar vertebra). A 10-g sample was taken from the anterior end of the LM section for calpastatin activity, and two 2.54-cm-thick chops were removed from the posterior end for slice shear force determination. The 3 muscles from the right sides were subsequently vacuum-packed and stored at 4°C until 7 d postmortem. At 7 d postmortem, 2 g of LM (from anterior end), IS, and SM (located such that the 24-h and 7-d samples were equally spaced across the longest dimension of the muscles) were taken and frozen in liquid nitrogen for caspase activity assays. Additionally, at 7 d postmortem, a 10-g sample was taken from the anterior end of the LM for calpastatin activity and two 2.54 cm-thick chops were removed from the posterior end for slice shear force determination. The LM was re-vacuum-packed and stored until 21 d postmortem and then sample collection for calpastatin, caspase activity, and slice shear force was repeated as for the 7-d samples.

Determination of Caspase Activity

Caspase 3/7 activity was measured in muscle samples using Promega's Apo-One Homogenous Caspase-3/7 Assay (Promega, Madison, WI), adapted for tissue samples (Wagner et al., 2003). Frozen muscle samples were crushed in liquid nitrogen, and 1 g was homogenized in 3 mL of extraction buffer [25 m*M* HEPES (pH 7.5), 0.1% (vol/vol) Triton X-100, 5 m*M* $MgCl_2$, 2 m*M* 1,4-dithiothreitol, 1.3 m*M* EDTA, 1 m*M* ethylene glycol tetraacetic acid, 15 μ*M* pepstatin, 20 μ*M* leupeptin, 74 μ*M* antipain, 0.15 μ*M* aprotinin] for 2 × 20 s. The homogenate was centrifuged at 15,000 × *g* for 20 min at 4°C. The supernatant was removed for the assay and a 1:1 ratio of caspase reagent to sample supernatant was used. The reaction was incubated at room temperature for the optimum incubation time (based on preliminary experiments) of 7 h. The end-point fluorescence was measured at an excitation wavelength of 485 ± 20 nm and an emission wavelength of 530 ± 25 nm on a Wallac 1420 Victor^2 multilabel counter (EG&G Wallac, Turku, Finland).

Caspase 9 activity was measured using Promega's Caspase-Glo 9 Assay (Promega). Muscle samples were prepared as described above for the caspase 3/7 assay, and a 1:1 ratio of caspase reagent to sample supernatant was used. The reaction was incubated at room temperature for 30 min (based on preliminary experi-

Table 1. Analysis of variance of fixed effects

	Caspase 3/7		Caspase 9		Calpastatin		Slice shear force	
Main effect	df	*F*-value	df	*F*-value	df	*F*-value	df	*F*-value
Genotype	1	3.20†	1	0.51	1	239.07***	1	91.80***
Muscle	2	81.80***	2	38.39***	—	—	—	—
Time	3	34.19***	3	80.21***	3	73.10***	2	20.39***
Genotype × Time	3	0.44	3	0.73	3	4.80**	2	0.73
Genotype × Muscle	2	1.49	2	1.42	—	—	—	—
Muscle × Time	6	1.11	6	2.80*	—	—	—	—
Genotype × Muscle × Time	6	0.34	6	0.41	—	—	—	—

†$P < 0.1$. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

ments) and luminescence measured on a Wallac 1420 Victor^2 multilabel counter (EG&G Wallac).

Optimum incubation times for the assays were determined empirically, and all samples were assayed in duplicate. The amount of luminescence or fluorescence generated is directly proportional to the amount of caspase activity present in the sample. Protein concentrations of sample supernatants used in the activity assays were determined using a Bradford assay (Bio-Rad, Hercules, CA).

Heated (Crude) Calpastatin

Calpastatin activity of fresh LM muscle was determined at 0, 2, 7, and 21 d postmortem as described by Koohmaraie et al. (1995). Calpastatin activity at 0 and 2 d from 4 callipyge and 4 normal lambs (2 ewes and 2 wethers from each genotype) were lost due to laboratory error. In brief, 10 g of minced LM sample was homogenized in 3 vol of extraction buffer (50 m*M* Tris-HCl, 10 m*M* EDTA, 2 m*M* phenylmethanesulfonylfluoride, 100 mg/L of ovomucoid, 6 mg/L of leupeptin, pH 8.3), centrifuged at 37,500 × *g* for 2 h at 4°C, and the supernatant dialyzed. The dialysate was heated to inactivate calpain activity and centrifuged at 37,500 × *g* for 2 h at 4°C, and the calpastatin in the supernatant was purified using a DEAE-Sephacel column. Calpastatin activity was determined from the inhibition of purified m-calpain activity by the calpastatin extracted from the LM. Data are reported as units of calpastatin activity per gram of muscle; a unit is defined as the amount of calpastatin needed to inhibit 1 unit of m-calpain activity.

Slice Shear Force

At 2, 7, and 21 d postmortem, fresh LM chops were cooked on a belt grill to an internal temperature of 71°C. Slice shear force was determined as described by Shackelford et al. (2004).

Statistical Analysis

Data were analyzed as a randomized complete block with repeated measures using the MIXED procedure (SAS Inst. Inc., Cary, NC). The model included fixed effects of genotype, sex, time postmortem, muscle and the interactions between genotype × time, genotype × muscle, muscle × time, and genotype × muscle × time. Kill day was considered a block and, therefore, was included as a random effect. Time postmortem was treated as the repeated effect with an auto-regression covariance structure. Least squares means were generated for significant ($P < 0.05$) interactions or main effects not included in higher order interactions and separated using pairwise *t*-tests (the DIFF option). Pearson correlation coefficients were generated using the PROC CORR procedure of SAS.

RESULTS

Analysis of Variance

The ANOVA indicated that genotype, muscle, and time were significant sources of variation for most traits (Table 1) and that genotype and time were significant sources of variation for caspase activities within muscles (Table 2). Genotype was a significant source of variation for caspase and calpastatin activities and slice shear force. Within muscles, genotype was a significant source of variation for caspase 3/7 activity. Muscle was a significant source of variation for caspase 3/7 and caspase 9 activities. Time was a significant source of variation for all traits. The only significant interactions were genotype × time for calpastatin activity and muscle × time for caspase 9 activity.

Caspase 3/7 Activity

Caspase 3/7 activity was determined at 0 h, 4 h, 8 h, 24 h, 2 d, 7 d, and 21 d postmortem in LM and at 0 h, 8 h, 24 h, and 7 d postmortem in SM and IS muscles (fewer muscle sampling times were used in the SM and IS than the LM due to the size of the muscles). In each of the muscles sampled from both callipyge and normal lambs, there was a general decrease in caspase 3/7 activity across the postmortem storage period (Table 3). Caspase 3/7 activity was greater in IS muscle compared with LM and SM muscles at all equivalent time points across the postmortem storage period in callipyge and normal lambs ($P < 0.001$), except at 8 h in SM muscle

Table 2. Analysis of variance of fixed effects within muscles[1]

	LM		SM		IS	
Item	Caspase 3/7	Caspase 9	Caspase 3/7	Caspase 9	Caspase 3/7	Caspase 9
Genotype	7.84**	2.47	7.79**	2.67	0.00	0.60
Time	77.23***	72.29***	13.00***	34.47***	16.63***	31.95***
Genotype × Time	0.76	0.92	0.45	0.79	0.55	0.47

[1]SM = semimembranosus; IS = infraspinatus.
**$P < 0.01$. ***$P < 0.001$.

from normal lambs. There were no differences ($P > 0.05$) detected in caspase 3/7 activity between LM and SM muscles at any time point in callipyge lambs. However, in normal lambs, caspase 3/7 activity at 8 h in SM was greater than that determined in LM ($P < 0.05$).

Caspase 3/7 activity in the LM was numerically greater at each time point measured across the storage period in normal lambs compared with callipyge lambs (except for 24 h); however, only a trend for caspase 3/7 activity to be greater at 21 d in normal lamb than callipyge ($P = 0.0523$) was observed. In callipyge and normal lambs, caspase 3/7 activity in LM was greater at 0 h, 4 h, 8 h, 24 h, and 2 d than at 7 and 21 d ($P < 0.001$). In LM muscle from normal lambs, caspase 3/7 activity at 4 h and 8 h was greater than that detected at 2 d ($P < 0.001$), with activity peaking at 8 h postmortem, which was greater than at-death activity ($P < 0.001$). In callipyge lamb LM, peak caspase 3/7 activity occurred at 24 h postmortem, with activity at 4, 8, and 24 h all greater than that determined at 2 d ($P < 0.001$), and activity at 8 and 24 h also greater than at 0 h ($P < 0.05$).

In SM muscle, caspase 3/7 activity was greater at all time points sampled during the conditioning period with caspase 3/7 activity significantly greater at 8 h ($P < 0.05$) and a trend to be greater at 24 h ($P = 0.0841$) in normal lambs than callipyge. Caspase 3/7 activity in SM muscle from both normal and callipyge lambs was significantly greater at 0, 8, and 24 h than at 7 d ($P < 0.001$), with activity peaking at 8 h postmortem, which was greater than that detected at 24 h in normal lambs ($P < 0.01$) and a trend to be greater in callipyge lambs ($P = 0.0678$).

Caspase 3/7 activity was greater at 0, 8, and 24 h than at 7 d postmortem in IS muscle from normal and callipyge lambs ($P < 0.001$). No significant differences in caspase 3/7 activity were detected between genotypes in IS muscle across the postmortem storage periods.

Caspase 9 Activity

Like caspase 3/7 activity, over the postmortem storage period there was a general decrease in caspase 9 activity in all 3 muscles sampled from callipyge and normal lambs (Table 4). Caspase 9 activity, like caspase 3/7 activity, also was greater in IS muscle than LM and SM muscles in callipyge and normal lambs at each equivalent time point ($P < 0.001$), except for 7 d and 8 h in SM muscle from normal lambs. In normal lambs, caspase 9 activity at 8 h in SM was greater than that measured in LM ($P < 0.05$); there were no other differences ($P < 0.05$) observed in caspase 9 activity between LM and SM in callipyge lambs. In LM from normal lambs, caspase 9 activity peaked at 8 h postmortem, whereas in callipyge lambs it peaked at 24 h. Caspase 9 activity was greater in LM sampled from both normal

Table 3. Mean ± SEM for caspase 3/7 activity across postmortem storage time in LM, semimembranosus (SM), and infraspinatus (IS) muscles from callipyge and normal lambs[1]

	LM		SM		IS	
Time, h	Callipyge	Normal	Callipyge	Normal	Callipyge	Normal
0	$761.83^{a,e}$ ± 42.85	$843.56^{c,e}$ ± 44.72	$947.71^{a,e}$ ± 55.43	$1{,}028.88^{c,e}$ ± 76.01	$1{,}531.43^{b,e}$ ± 174.80	$1{,}494.66^{d,e}$ ± 107.81
4	803.68^{ef} ± 24.32	898.43^{ef} ± 42.27	ND[2]	ND	ND	ND
8	$864.62^{a,f}$ ± 35.96	$954.93^{c,f}$ ± 61.71	$979.82^{a,e}$ ± 58.82	$1{,}225.14^{d,e}$* ± 167.95	$1{,}432.25^{b,e}$ ± 187.82	$1{,}417.67^{d,e}$ ± 131.45
24	$883.42^{a,f}$ ± 70.33	$853.39^{c,ef}$ ± 48.33	$784.67^{a,e}$ ± 39.91	$970.52^{c,f}$† ± 89.37	$1{,}343.05^{b,e}$ ± 176.67	$1{,}500.38^{d,e}$ ± 207.55
48	687.15^{g} ± 46.60	760.21^{e} ± 49.09	ND	ND	ND	ND
168	$319.39^{a,h}$ ± 35.54	$375.82^{c,g}$ ± 33.50	$522.23^{a,f}$ ± 109.26	$654.02^{c,g}$ ± 94.01	$1{,}007.46^{b,f}$ ± 104.45	$918.16^{d,f}$ ± 99.56
504	161.80^{h} ± 30.14	275.60^{g}† ± 61.56	ND	ND	ND	ND

[a,b]Means between muscles within callipyge lambs lacking a common superscript differ ($P < 0.01$).
[c,d]Means between muscles within normal lambs lacking a common superscript differ ($P < 0.05$).
[e–h]Means within columns lacking a common superscript differ ($P < 0.05$).
[1]Arbitrary units of fluorescence/micrograms of protein.
[2]ND = not determined.
†Trend for means between genotypes to be different ($P < 0.10$).
*Means between genotypes are different for SM at 8 h ($P < 0.05$).

Table 4. Mean ± SEM for caspase 9 activity across the postmortem storage period in LM, semimembranosus (SM), and infraspinatus (IS) muscles from callipyge and normal lambs[1]

	LM		SM		IS	
Time, h	Callipyge	Normal	Callipyge	Normal	Callipyge	Normal
0	3,175.01^{a,f} ± 290.02	3,047.55^{c,f} ± 298.60	3,459.49^{a,f} ± 306.68	3,623.73^{c,f} ± 379.59	5,597.04^{b,f} ± 631.87	6,261.46^{d,f} ± 975.62
4	3,181.83^{f} ± 414.82	3,149.54^{fg} ± 308.72	ND[2]	ND	ND	ND
8	3,922.26^{a,g} ± 334.77	3,783.03^{c,g} ± 291.53	3,980.91^{a,f} ± 366.56	4,905.99^{d}* ± 659.58	6,163.55^{b,f} ± 714.13	6,439.30^{e,f} ± 960.91
24	4,031.98^{a,g} ± 504.68	3,135.46^{c,fg} ± 402.05	3,482.04^{a,f} ± 346.66	3,864.43^{c,f} ± 545.26	5,132.88^{b,f} ± 677.72	4,727.85^{d,g} ± 652.05
48	1,821.08^{h} ± 199.27	1,602.76^{h} ± 69.76	ND	ND	ND	ND
168	646.20^{a,i} ± 85.76	552.13^{c,i} ± 49.77	902.78^{a,g} ± 224.69	898.32^{c,h} ± 140.60	963.02^{a,g} ± 179.46	674.23^{c,h} ± 125.85
504	484.42^{i} ± 46.47	537.16^{c,i} ± 85.80	ND	ND	ND	ND

^{a,b}Means between muscles within callipyge lambs lacking a common superscript differ ($P < 0.001$).
$^{c–e}$Means between muscles within normal lambs lacking a common superscript differ ($P < 0.001$).
$^{f–i}$Means within columns lacking a common superscript differ ($P < 0.05$).
[1]Arbitrary units of luminescence/microgram of protein.
[2]ND = not determined.
*Means between genotypes are different for SM at 8 h ($P < 0.05$).

and callipyge lambs at 0, 4, 8, and 24 h than 2, 7, and 21 d ($P < 0.0001$), with activity at 2 d also greater than at 7 and 21 d ($P < 0.01$). In LM from normal lambs, caspase 9 activity at 8 h was greater than at 0 h ($P < 0.05$) and a trend to be greater than at 24 h ($P = 0.0782$). Similarly, caspase 9 activity in LM from callipyge lambs was greater at 8 and 24 h than at 0 and 4 h ($P < 0.05$).

In SM muscle, caspase 9 activity was greater in normal lambs than callipyge at 8 h postmortem ($P < 0.05$). Caspase 9 activity was greater at 0, 8, and 24 h than at 7 d in normal and callipyge lambs ($P < 0.0001$), with caspase 9 activity at 8 h also greater than that measured at 0 and 24 h in normal lamb SM muscle ($P < 0.05$).

Like caspase 9 activity in SM, caspase 9 activity at 0, 8, and 24 h was greater than that at 7 d in IS muscle from both callipyge and normal lambs ($P < 0.0001$). Caspase 9 activity at 24 h also was less than at 0 and 8 h in IS from normal lambs ($P < 0.05$). There were no differences in caspase 9 activity in IS between genotypes at any of the time points across the storage period. Caspase 9 activity was found to positively correlate to caspase 3/7 activity in LM, SM, and IS muscles across the whole time course [r = 0.85, r = 0.86, r = 0.84, respectively ($P < 0.0001$)], and at each individual time point sampled ($P < 0.001$) within muscles.

Calpastatin Activity

Calpastatin activity was determined at 0 h, 2 d, 7 d, and 21 d postmortem in the LM (Table 5) and was greater in the callipyge lambs compared with normal lambs at each time point measured ($P < 0.001$). The rate of decrease in LM calpastatin activity during postmortem storage was faster in normal lambs compared with callipyge lambs. In callipyge lambs, calpastatin activity was greater at 0 and 2 d than that measured at 7 and 21 d ($P < 0.001$), with activity at 0 d also greater than that determined at 2 d ($P < 0.0001$). Calpastatin activity in normal lambs was greater at 0 d than that measured at 2, 7, and 21 d ($P < 0.0001$), and no differences were detected between 2, 7, and 21 d calpastatin activity.

In normal lamb, LM caspase 3/7 activity peaked at 8 h postmortem and a negative correlation was detected between peak caspase 3/7 activity and calpastatin activity at 0 d and 2 d (r = −0.65, r = −0.68, respectively, $P < 0.05$). This relationship was not detected in LM from callipyge lambs.

Slice Shear Force

Slice shear force values of the LM were greatest at 2 d and least at 21 d. Slice shear force values for the LM were greater ($P < 0.0001$) in callipyge lambs compared with normal lambs (Table 6), indicating limited postmortem proteolysis and tenderization in callipyge lambs relative to normal lambs. The interaction of genotype × day was not significant ($P = 0.49$) for LM slice shear force. A positive correlation between slice shear force and calpastatin activity across the postmortem storage period was detected ($P < 0.0001$, r = 0.82).

Table 5. Mean ± SEM for calpastatin activity in LM of callipyge and normal lambs at 4 different time points during postmortem storage[1]

Day	Callipyge	Normal
0	3.20 ± 0.36^{a}*	1.51 ± 0.30^{a}*
2	1.77 ± 0.14^{b}*	0.48 ± 0.06^{b}*
7	1.22 ± 0.13^{c}*	0.23 ± 0.04^{b}*
21	1.03 ± 0.04^{c}*	0.20 ± 0.02^{b}*

$^{a–c}$Means within the same column with different superscripts are different ($P < 0.05$).
[1]Units are total calpastatin activity/gram of muscle.
*Means between genotypes are different ($P < 0.05$).

DISCUSSION

It is well established from a large volume of indirect evidence that the calpain system has a significant role in postmortem proteolysis and meat tenderization (Koohmaraie and Geesink, 2006). However, 2 recent studies provided the first direct evidence of the calpain system in postmortem proteolysis. Kent et al. (2004) demonstrated that in transgenic mice, overexpressing calpastatin postmortem proteolysis was severely attenuated. The 80 kDa subunit of μ-calpain declined in the transgenic mice and control mice; however, the rate of decrease was slower in transgenic mice, indicating that overexpression of calpastatin slowed down μ-calpain autolysis rather than completely stopped it. Additionally, degradation of μ-calpain substrates was reduced. In transgenic mice, 83% of at-death desmin was retained after 7 d of postmortem storage compared with 9% in normal mice. From this study, the authors concluded that overexpression of calpastatin leads directly to a decrease in μ-calpain proteolytic activity as shown by reduced autolysis and decreased degradation of its substrates. Similarly, Geesink et al. (2006) showed that postmortem proteolysis of key myofibrillar proteins including nebulin, desmin, dystrophin, and troponin-T was severely reduced in muscle from μ-calpain knockout mice compared with wild type and that these degradation patterns were similar to those observed by Kent et al. (2004) in mice overexpressing calpastatin. Geesink et al. (2006) propose that further research should focus on the regulation of μ-calpain in postmortem proteolysis.

The callipyge phenotype was first identified in a Dorset ram that transmitted a heavy muscling phenotype to some of his offspring. The callipyge gene arises from a genetic mutation in chromosome 18 (Freking et al., 1998) that results in postnatal muscle hypertrophy (Koohmaraie et al., 1995; Freking et al., 1999). An SNP in an intergenic region of the delta drosphila-like homolog 1 (**DLK1**)/maternally expressed gene 3 (**MEG3**) imprinted domain has since been identified to be responsible for the callipyge phenotype (Freking et al., 2002). In agreement with Delgado et al. (2001), this study has shown that calpastatin activity in LM sampled from callipyge lambs was greater than that from normal lambs and calpastatin activity decreased faster in normal lambs than callipyge. The extent of tenderization, as determined by slice shear force was less in LM from callipyge than normal lambs, with slice shear force values greater in callipyge LM at each time point across the postmortem storage period. These results concur with previous studies (Koohmaraie et al., 1995; Geesink and Koohmaraie, 1999; Duckett et al., 2000; Delgado et al., 2001) and add to the overwhelming evidence that elevated calpastatin content inhibit calpain activity and, therefore, reduce postmortem proteolysis and tenderization in LM from callipyge lamb.

There is a growing body of literature demonstrating cross-talk between the calpain and the caspase proteolytic systems. The objective of this study was to investigate whether there is a potential role for the caspase proteolytic system in postmortem proteolysis and meat tenderization and to examine any interactions between these 2 protease families, using the callipyge model. Caspase 9 is an initiator caspase involved in the intrinsic or mitochondrial-mediated apoptotic pathway and is activated by environmental stress such as hypoxia and ischemia (Earnshaw et al., 1999). Caspases 3 and 7 are executioner or effector caspases and are activated by upstream initiator caspases such as caspase 9, and once activated, they target and cleave their specific substrates (Boatright et al., 2003). In this current study, caspase 3/7 activity and caspase 9 activity decreased in all 3 muscles (LM, SM, and IS) sampled across the postmortem conditioning period in callipyge and normal lambs. The detection of caspase activity and the changes in activity suggests that caspases are active during the postmortem conditioning period. Caspase 9 activity was found to decrease faster than caspase 3/7 activity with approximately 22% of at-death activity detected at 7 d compared with 55%. In conjunction with the positive correlation observed between the initiator and executioner isoforms, this suggests that caspase 9 is responsible for the activation of caspases 3/7 and that these changes correspond to the cascade of events that occur in situ.

These changes in caspase activities across the postmortem storage period and the relationship between caspase 9 and caspase 3/7 are in agreement with previous findings in pork LM (Kemp et al., 2006). However, the rate of decline in caspase activity was much more rapid in pork LM with approximately only 6% of at-death activity remaining at 8 d. This is consistent with

Table 6. Least squares means for slice shear force in LM from normal and callipyge lambs

Item	Slice shear force, kg
Main effect	
Genotype	
Normal	15.70[b]
Callipyge	37.53[a]
P-value	0.0001
Day	
2	33.18[a]
7	25.96[b]
21	20.71[c]
P-value	0.0001
Interaction	
Normal	
d 2	22.90
d 7	13.98
d 21	10.23
Callipyge	
d 2	43.46
d 7	37.93
d 21	31.19
P-value	0.49

[a–c]Means within main effect lacking a common superscript differ ($P < 0.05$).

the observations that pork tenderizes faster than lamb (Koohmaraie et al., 1991) and less storage time is required to obtain the benefits of postmortem storage on meat tenderness in pork relative to lamb (Koohmaraie, 1996).

The callipyge genotype is known to affect numerous muscles throughout the carcass depending on the content of calpastatin in that muscle and the extent callipyge increases that level of calpastatin (Koohmaraie et al., 1995). In this study, the LM, SM, and IS muscles were sampled because of differences in the extent that the callipyge genotype affects them. Muscles of the pelvic limb such as SM, semitendinosus biceps femoris, gluteus medius and gracilis, and the loin region of the carcass such as the psoas and LM undergo substantial muscle hypertrophy in the callipyge phenotype. However, the majority of muscles from the thoracic region, including the supraspinatus, IS, and lateral digital extensor, do not hypertrophy (Koohmaraie et al., 1995; Jackson et al., 1997b; Duckett et al., 2000), exhibit myofiber type changes (Carpenter et al., 1996), or differ in calpastatin activity in callipyge relative to normal animals (Koohmaraie et al., 1995). Additionally, LM, SM, and IS have different metabolic, contractile, and physical properties, which all influence meat quality. The LM and SM are classified as fast glycolytic white muscles, whereas the IS is a slow oxidative red muscle. Proteolysis occurs faster in fast glycolytic white muscles than in slow oxidative red muscles (Koohmaraie et al., 1988; Monin and Ouali, 1991; Whipple and Koohmaraie, 1992) and calpastatin activity has been shown to be less in fast glycolytic white muscles than red oxidative slow muscles (Ouali and Talmont, 1990; Whipple and Koohmaraie, 1992; Sazili et al., 2005). In the LM, the proteolytic capacity is the major determinant of tenderness (Koohmaraie et al., 2002), SM has less proteolytic potential than LM (Wheeler et al., 2000; Rhee et al., 2004), and connective tissue content is the major determinant of SM tenderness (Rhee et al., 2004). In the LM and also the SM, muscles that are both affected by the callipyge gene, caspase 3/7 activity was greater in normal lambs than callipyge lambs at 8 h postmortem in SM with a trend to be greater at 24 h in SM and at 21 d in LM. This pattern also was detected for caspase 9 activity in SM muscle where activity at 8 h postmortem was greater in normal lambs than in callipyge lambs. The rate of caspase 3/7 activity decrease in LM was faster than that detected in SM, 44% of at-death activity compared with 61% at 7 d, presumably as caspases 3/7 were cleaving their targeted substrates. This is consistent with previous reports that LM has a greater proteolytic capacity than SM (Wheeler et al., 2000, Rhee et al., 2004).

In the IS muscle from normal lambs, calpastatin activity is already increased, and the IS, therefore, is not affected by the callipyge phenotype (Koohmaraie et al., 1995; Delgado et al., 2001). The IS from callipyge and normal lambs undergoes similar extent of postmortem tenderization, as determined by myofibrillar fragmentation index, and the extent of tenderization was less than that observed in the biceps femoris and LM from normal sheep, indicating less postmortem proteolysis (Delgado et al., 2001). In contrast to LM and SM, there was no effect of genotype on caspase activities in the IS, with caspase 3/7 and caspase 9 activity in IS greater than that detected in SM and LM. This could, therefore, suggest that the caspase proteolytic system is in some way affected by the callipyge gene. A potential explanation is an interaction between the 2 protease systems. Caspases have been shown to indirectly upregulate calpain activity through cleavage of calpastatin (Porn-Ares et al., 1998; Wang et al., 1998). Furthermore, inhibition of caspases has been demonstrated to increase calpastatin expression (Sun et al., 2008). A negative correlation was observed between caspase 3/7 and calpastatin activity at each time point sampled for both genotypes. However, only the relationships between calpastatin activity at 0 and 2 d and peak caspase 3/7 activity in normal sheep were statistically significant, such that the greater the caspase 3/7 activity the less the calpastatin activity. This could suggest that caspases may contribute to the decrease in calpastatin in normal lamb, but its activity is not sufficient to exert any effect on the elevated calpastatin content in callipyge lamb. Thus, it is the abundance of substrate that has changed rather than the overall content of caspases or calpains. Additionally, if caspases were affecting postmortem proteolysis independently of the calpain system, then it would be expected that tenderization would proceed independently of the increased calpastatin abundance. This study and others have shown that this does not happen; however, an interaction effect between the systems cannot be excluded.

In addition to increased calpastatin activity and muscle mass, muscles affected by the callipyge phenotype have a greater muscle DNA and RNA content, suggesting that these muscles have an increased capacity for protein synthesis (Koohmaraie et al., 1995). Lavulo et al. (2008) demonstrated that myoblasts isolated from callipyge LM and semitendinosus muscles were more resistant to apoptosis than those isolated from those muscles of normal lambs. Apoptosis was determined by fluorescent activated cell sorting analysis, and although caspase activity was not measured directly, caspases are responsible for apoptosis, and therefore, it is fair to speculate that in apoptosis-resistance myoblasts isolated from callipyge muscles there could be less caspase activity. The mechanism behind these results are unknown; however, the authors suggested that the decreased sensitivity of the myoblasts to pro-apoptotic signals in the LM and semitendinosus from the callipyge sheep could result in increased numbers of myogenic cells or satellite cells. This pool of quiescent mononucleated myogenic cells is thought to be the source of nuclei in hypertrophying muscle (Lavulo et al., 2008), and it is speculated that increased proliferation of satel-

lite cell-derived myoblasts in affected muscles may contribute to the callipyge phenotype (Koohmaraie et al., 1995).

Recently, the gene DNAJA1 has been identified to be downregulated in longissimus thoracis muscle from Charolais bulls with increased meat quality using micro-arrays. Its expression was shown to inversely relate to tenderness and explained up to 63% of the variation observed (Bernard et al., 2007). The DNAJA1 gene encodes a member of the heat shock protein family (**Hsp40**), which co-chaperones the 70 kDa heat shock protein (**Hsp70**). The DNAJA1/Hsp70 complex has been shown to directly inhibit apoptosis by preventing the pro-apoptotic protein Bax to translocate to the mitochondrial membrane, where it undergoes conformational changes triggering the release of cytochrome c, an essential component in activating the intrinsic caspase 9 pathway (Gotoh et al., 2004). Bernard et al. (2007) suggested that if apoptosis and caspases are involved in postmortem proteolysis, then the reduced anti-apoptotic activity of downregulated DNAJA1 could facilitate cell death in the postmortem period and, consequently, increase tenderization. However, this study was performed in cattle and the use of micro-arrays is a relatively new tool for identifying markers of meat quality; therefore, further research is needed to understand the relationships between gene expression and meat quality.

The aim of this study was to determine if there is a role for the caspase proteolytic system in postmortem proteolysis and meat tenderization. μ-Calpain knockout studies (Geesink et al., 2006) and inhibition of μ-calpain through overexpression of calpastatin (Kent et al., 2004) has shown that postmortem proteolysis is severely attenuated and provides direct evidence that μ-calpain is indeed primarily responsible for postmortem proteolysis and meat tenderization. However, in both of these studies some postmortem proteolysis was still detected. The authors attributed this limited proteolysis to autolysis of m-calpain that was detected in murine skeletal muscles; nonetheless, autolysis of m-calpain does not occur in ovine or bovine muscles. Studies by Koohmaraie et al. (1987), Geesink and Koohmaraie (1999), and Veiseth et al. (2001) have demonstrated that m-calpain is incredibly stable in muscle postmortem and autolysis is only detectable when calcium concentrations are elevated.

Currently, the evidence to show that caspases may be involved in postmortem proteolysis and meat tenderization is indirect. Caspases have been shown to be active during the postmortem storage period, that their activity in the early conditioning period correlates to shear force (Kemp et al., 2006), and that incubation of myofibrils with recombinant caspase 3 produces the same proteolytic pattern as observed in postmortem muscle (Kemp and Parr, 2008). Koohmaraie (1988) specifies for a protease system to be involved in postmortem proteolysis and meat tenderization, it must be endogenous to skeletal muscle, it must have access to myofibrils in tissue, and it must be able to mimic postmortem changes in myofibrils. The caspase proteolytic system fulfils these requirements, and it is, therefore, not possible to rule out that caspases could contribute to the limited postmortem proteolysis observed in the Kent et al. (2004) and Geesink et al. (2006) studies. Caspases could have a role in tenderization through modulating the calpain system, most likely by degrading the calpain specific inhibitor calpastatin. Studies by Porn-Ares et al. (1998) and Wang et al. (1998) demonstrated that caspases are capable of cleaving calpastatin resulting in an increase in calpain activity. In this current study, an effect of genotype on caspase 3/7 was observed in muscles that are hypertrophied in the callipyge genotype but not in a muscle that is not hypertrophied by the callipyge. A negative correlation was detected between calpastatin activity at 0 and 2 d and peak caspase 3/7 activity. This evidence for caspase involvement in postmortem proteolysis and meat tenderization is indirect, although other studies suggest a role for caspases and apoptosis in postmortem proteolysis. Therefore, it is not possible to confirm or reject a role of this protease system in postmortem proteolysis and meat tenderization.

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